IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, Florian Gunzer, MD, hereby state:

1. I am a medical microbiologist at Hannover Medical School in Hannover, Germany. I currently work as a research scientist, focused on elucidating virulence mechanisms of Shiga toxin producing *Escherichia coli* (STEC) and enterohemorrhagic *Escherichia coli* (EHEC) causing human disease. I am using *in vitro* systems such as tissue culture and array analysis as well as animal models for *in vivo* investigation. I have published my work in several peer reviewed international scientific papers. I do also have an appointment as infectious disease consultant with the department of pediatrics at Hannover Medical School.

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2. I have no interest in the above-identified patent application nor have I been compensated for my time.

3. I have worked since 1994 with the swine model for human infection with enteric pathogens. The neonatal gnotobiotic colostrum deprived piglet is uniquely relevant as a model to study human infections with O157 and non-O157 STEC/EHEC. In humans, oral infection with STEC/EHEC may cause severe enteritis with bloody diarrhea and, in certain circumstances, hemorrhagic colitis, followed in up to 10 % of cases by an extraintestinal complication, the hemolytic uremic syndrome (HUS). Hemolytic uremic syndrome is characterized through a triad of symptoms, anemia, thrombocytopenia, and acute renal failure due to vascular lesions, described as thrombotic microangiopathy (TMA). Thrombotic microangiopathy is the morphological hallmark of all forms of hemolytic uremic syndrome. STEC/EHEC produces several virulence factors among which a family of phage encoded cytotoxins, called Shiga toxin 1 or Shiga toxin 2, appears to be most important. Enteric manifestations of STEC/EHEC infection are attributed in part to the attaching and effacing (A/E) phenotype of the pathogens. Formation of A/E lesions requires expression of genes encoded by the LEE (locus of enterocyte effacement) region in the STEC/EHEC genome. The intimate enterocyte attachment of these organisms is thought to be critical for intestinal colonization and in facilitating transport of Shiga toxins from the intestine into the bloodstream where (in a proportion of patients) it

causes the systemic vascular renal and neurologic damage characteristic of HUS.

4. In a recent publication from my laboratory (F. Gunzer et al., Am.J.Clin.Pathol. 2002, 118:364-375) we could show for the first time, that neonatal gnotobiotic colostrum deprived piglets developed renal TMA, the hallmark of HUS in humans, following infection with either an O157:H7 or an O26:H11 EHEC strain. In addition to these vascular alterations, we observed A/E lesions in the gut and microhemorrhages in the CNS, pathologic changes that had been described by other investigators before. The clinical response of gnotobiotic piglets very closely resembled intestinal and extraintestinal features of human EHEC disease. After oral uptake of the pathogens, the natural route of infection, the animals developed diarrhea during a prodromal period, followed by transport of Shiga toxin to the bloodstream in sufficient quantities to cause systemic vascular damage, clinically apparent systemic manifestation of disease and death

5. For the above reasons neonatal gnotobiotic colostrum deprived piglets have a unique potential as a model to evaluate prophylactic or therapeutic approaches offering new advantages to prevent or lessen systemic complications of EHEC infection in humans.

6. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the

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knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Florian Gunzer, MD

Date: April 1, 2003

ATL1 #569711 v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, John M. Leong, M.D., Ph.D., hereby state:

1. I am Associate Professor of Molecular Genetics and Microbiology at the University of Massachusetts Medical School. I am a former Pew Scholar in the Biomedical Sciences and a former Established Investigator of the American Heart Association. I have published numerous papers in peer reviewed journals, including an invited commentary in 2002 on a toxin produced by the enteric pathogen *Campylobacter jejuni* in the journal Science and a 2003 review on colonization by enterohemorrhagic *E. coli* O157:H7 in the journal Current Opinions in Microbiology.

2. I have no interest in the above-identified patent application nor have I been compensated for my time.

3. The life-threatening complications of human infection by Shiga Toxin Producing Strains of *E. coli* 0157:H7 (STEC) are hemorrhagic colitis and the triad of hemolytic anemia, thrombocytopenia, and kidney failure known as hemolytic uremic syndrome (HUS). In my expert opinion, the features that are central to the ability of STEC to cause this local and systemic damage are the ability to: (1) secrete shiga-like toxin, which contributes to intestinal damage and, through its toxicity to vascular endothelium, is essential for the systemic manifestations of STEC infection, and (2) generate attaching and effacing (AE) lesions on the intestinal epithelium, lesions that disrupt the cytoskeleton of epithelial cells. This disruption compromises intestinal epithelial integrity and is likely to promote the systemic absorption of shiga-like toxin produced by bacteria in the gut.

4. The neonatal gnotobiotic piglet is the only animal model for infections with *E. coli* O157:H7 and other serotypes of STEC that reproduces both of these critical elements of STEC pathogenesis. STEC O157:H7 generate AE lesions on intestinal epithelium, cause hemorrhagic colitis and systemic damage, mainly neurological via vascular damage in the central nervous system by shiga like toxin absorbed from the gut. (Some small animals, e.g. the mouse, are susceptible to systemic effects of shiga-like toxin, but do not manifest AE lesions upon intestinal infection—thus, some bacterial mutants

that are incapable of causing disease in humans are likely to be fully virulent in these animals.) As such, the gnotobiotic piglet is the best model for evaluating therapies for the prevention or treatment of tissue damage by STEC infection, in particular systemic manifestations due to endothelial damage by shiga-like toxin. Accurate estimation of the efficacious dose(s) of prophylactic or therapeutic agent to be administered to human patients, and the timing of those doses, is best determined in gnotobiotic piglets.

5. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

John M. Leong, M.D, Ph.D.

Date: 3/27/03

ATL1 #569711 v1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Saul Tzipori, Ramaswamy Balakrishnan and Arthur Donohue-Rolfe

Serial No.: 10/041,958 Art Unit: 1645

Filed: January 2, 2002 Examiner: Mark Navarro

For: *HUMAN NEUTRALIZING ANTIBODIES AGAINST HEMOLYTIC UREMIC SYNDROME*

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.132

Dear Sir:

I, Saul Tzipori, DVM, PhD, DSc, FRCVS, hereby state:

1. I am a Professor of Microbiology and Head of the Division of Infectious Diseases, at Tufts University School of Veterinary Medicine in Massachusetts.

3. I have conducted original scientific research on the prevention of systemic complications in *Escherichia coli* O157:H7 infection over the last two decades, using all the known laboratory techniques and the currently existing animal models including the mouse and the piglet models.

4. Diarrhea followed by systemic disease occurs only in humans and pigs when infected with the *E. coli* bacteria that produce Shiga toxin or Stx. The bacteria induce serious damage to the gut, which results in diarrhea in both humans and piglets. The Stx which is liberated by the bacteria in the gut is absorbed from the damaged gut in humans and piglets into the blood stream where it can damage blood vessels. In humans this

damage can lead to hemolytic uremic syndrome (HUS) manifested as kidney failure. In piglets, absorbed Stx causes diarrhea and brain damage. The only animal that has the receptors required for absorption of Stx is the piglet. Therefore piglets are the only model which can be used to determine the therapeutic dose against the systemic effect of the Stx. This includes the amount of human monoclonal antibody against Stx required to protect patients presenting with HUS, or diarrhea, or infected with, or exposed to the Stx-producing *Escherichia coli* bacteria. No other animal models including mice develop damaged gut and diarrhea after infection.

5. We consistently protect piglets experimentally infected with the bacteria well after they develop diarrhea, and before the onset of the brain injury and neurological symptoms. This mimics the situation in patients who can similarly be treated with the antibody after they present with diarrhea and before the onset of HUS which occurs 4-6 days later. We have determined that 5micrograms of Stx antibody must be present in each ml of blood to fully protect a single piglet from developing neurological symptoms and death. This requires a dose of 3mg of antibody per each kg of body weight. Based on these experiments, patients presenting with diarrhea, will similarly require to have 5 micrograms/ml of antibody circulating in their blood to be fully protected against the development of HUS. The exact injectable dose required to establish this amount of antibody in the blood stream of human individuals will be determined in a dose-response study during phase I clinical trials.

6. Given the incidence of HUS in the population, without studies in piglets it will take 10-12 years to determine the effective dose through Phase II/III clinical trials in humans. These bacteria do not cause gut damage or diarrhea in other animals including

the mouse model which is used by other investigators. The lack of gut damage and diarrhea reduce considerably the susceptibility of mice to Stx, and consequently alters the amount of antibody needed to protect them. The relative amount needed to protect a mouse will therefore be very different than that needed to protect a more susceptible host such as humans or piglets.

7. I have reviewed U.S. patent No. 5,512,282 to Krivan, et al. Krivan does not describe a method of preventing HUS in humans. Krivan et al describe the oral administration of polyclonal antibodies produced in cattle which are suitable for treating Stx-related diseases in animals. Unquestionably, polyclonal antibodies made in animals, however purified, cannot be injected into the blood stream of humans, either for treatment or prevention. More importantly, there is no evidence that specific antibodies, be it polyclonal or monoclonal, are effective at all when given orally, nor that they can prevent or protect against a systemic disease caused by toxin present in the blood stream. They will be digested and metabolized in the gut, even when the antibodies are administered either encapsulated, conjugated or emulsified. While they provide many examples to show how these polyclonal antibodies may be useful as diagnostic reagents for the detection of toxins in food products or stool, they provide no evidence what so ever as to how the administration of such antibody might safely and effectively protect, ameliorate, or prevent Stx-mediated systemic disease.

8. Attached are letters written by two experts in this field, Dr. Harley W. Moon of the College of Veterinary Medicine at Iowa State University, and Dr. Phillip I. Tarr, Professor of Pediatrics and Microbiology, Washington University School of Medicine in

Serial No.: 10/041,958
Filed: January 2, 2002
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St. Louis, in support of the unique role of the pig model in testing agents and determining the effective dosages for the treatment or prevention of HUS.

9. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

S. Tzipori

Saul Tzipori, DVM, PhD, DSc, FRCVS

Professor of Microbiology

Date: 4/10/03

ATL1 #570386 v1

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

College of Veterinary Medicine
Department of Veterinary Pathology
Ames, Iowa 50011-1250
515 294-3282
FAX 515 294-5423

March 12, 2003

To Whom It May Concern:

Subject: Swine Model for Human Infection with Shiga Toxin Producing (STEC) Strains of *E. coli* O157:H7

The neonatal gnotobiotic or colostrum deprived piglet is uniquely relevant as a model for human infections with *E. coli* O157:H7 and other serotypes of STEC.
In humans, *E. coli* O157:H7 causes intestinal infection and diarrheal disease leading in some cases to hemorrhagic colitis. These enteric manifestations of the infection are attributed in part to the intestinal attaching and effacing attribute of the pathogen. This attribute requires expression of LEE (locus of enterocyte effacement) region genes of the STEC. Expression of LEE region genes and the resulting intestinal lesions are thought to be critical for intestinal colonization, production of clinically significant amounts of Shiga toxin in the intestine and in facilitating transport of Shiga toxin from the intestine into the blood where (in a proportion of patients) it causes the systemic vascular, renal and neurologic damage characteristic of the Hemolytic Uremia Syndrome.

Neonatal gnotobiotic or colostrum deprived piglets are the only animal model I am aware of wherein LEE region dependent colonization by *E. coli* O157:H7 results in attaching/effacing colonic lesions and diarrhea during a prodromal period, followed by transport of Shiga toxin to blood in sufficient quantities to cause systemic vascular damage, clinically apparent systemic manifestation of disease and death.

For the above reasons neonatal gnotobiotic or colostrum deprived pigs have a unique potential as a model to evaluate prophylactic or therapeutic approaches to human STEC infections.

Sincerely,

Harley W. Moon
Professor

Washington University Physicians
Washington University School of Medicine in St. Louis

Children's
HOSPITAL • ST. LOUIS

Research Units
Developmental Biology Unit
Cell and Molecular Biology Unit
Infection, Immunity & Inflammation Unit
Patient Oriented Research Unit

19 March 2003

To Whom It May Concern:

I am writing to render an unsolicited opinion regarding the prevention of systemic complications in *Escherichia coli* O157:H7 infection. I write from the position of a physician and researcher who has studied hundreds of children with this infection, a subset of whom who have developed hemolytic uremic syndrome (HUS).

Our data indicate that prothrombotic coagulation abnormalities are well underway early in illness (by day four of illness), well in advance of the development of HUS. Specifically, fibrinolysis is inhibited, and thrombin is being generated, and there is evidence for intravascular fibrin accretion in infected patients well before there is renal insufficiency, or renal tubular injuries (Chandler WL, et al. N Engl J Med 2002; 346:23).

Additionally, there is evidence in some infected patients for the fragmentation of circulating von Willebrand factor (Tsai HM, et al. Pediatr Res 2001; 49:653). Antibiotic administration has not been demonstrated to provide any benefit to infected children, and considerable data to suggest that such therapy actually increases the risk of developing HUS (Wong CS, et al. N Engl J Med 2000; 342:1930). Accordingly, it is my belief that if any toxin interdiction technologies are to work, they must be administered in the pre-symptomatic phase (following ingestion, prior to the first loose stool), or early in illness (as soon as diarrhea begins). The administration of such therapeutics after a culture is positive, would take place during a phase of illness when it is likely that the vascular insult would have already occurred.

To test whether such products given as soon as diarrhea begins are likely to be effective, an animal model is required which develops diarrhea well before the onset of systemic complications are apparent.

Yours sincerely,

Phillip I. Tarr, M.D.
Professor of Pediatrics and Microbiology

PIT/jfm

Washington University School of Medicine at
Washington University Medical Center,
McDonnell Pediatric Building
Campus Box 8208, 660 South Euclid Avenue

APPENDIX OF RELATED PROCEEDINGS

There is a related appeal in Serial No: 10/230,614 filed August 29, 2002, which directly affects, which would be directly affected by, or which may have a bearing on the Board's decision in this appeal. A decision was rendered by the Board of Patent Appeals on September 26, 2005. A copy of the decision is enclosed.

TO: TSICON PTO

The opinion ... support of the decision being entered today ... was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte SAUL TZIPORI, RAMASWAMY BALAKRISHNAN, and ARTHUR DONOHUE-ROLFE

Appeal No. 2005-1921
Application No. 10/230,614

ON BRIEF

Before SCHEINER, ADAMS, and GREEN, Administrative Patent Judges.

ADAMS, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on the appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 1, 4-6, and 27-32, which are all the claims pending in the application.

Claim 1 is illustrative of the subject matter on appeal and is reproduced below:

1. A method to prevent or treat hemolytic uremic syndrome in a human individual exposed to or infected by Escherichia coli producing Shiga-like toxin II, comprising:
administering intradermally, subcutaneously, intravenously, or intramuscularly, to an individual presenting with bloody diarrhea, diagnosed with infection by Escherichia coli producing Shiga-like toxin II, or exposed to an individual infected with or exposed to the same source of infection with Escherichia coli producing Shiga-like toxin II

Docketed for 11-26-05 Request Rehearing / Appeal Federal Court Due Drop Dead Date
By:

> an effective amount of monoclonal human or humanized antibodies consisting of antibodies neutralizing Shiga like toxin II, to prevent or treat hemolytic uremic syndrome.

The references relied upon by the examiner are:

Krivan et al. (Krivan)	5,512,282	Apr. 30, 1996
Queen et al. (Queen)	WO 90/07861	Jul. 26, 1990

Engleman et al. (Engleman), Human lymphoblastoid Cell Lines as Fusion Partners, in Human Hybridomas and Monoclonal Antibodies, pp. 23-27 (Edgar G. Engleman et al., eds., Plenum Press, New York 1985)

MacLeod et al. (MacLeod), "Immunization of pigs with a purified Shiga-like toxin II variant toxoid," Veterinary Microbiology, Vol. 29, pp. 309-318 (1991)

GROUND OF REJECTION

Claims 1, 4-6, and 27-32 stand rejected under 35 U.S.C. § 103, as being unpatentable over the combination of Krivan, MacLeod, Queen and Engleman.

CLAIM GROUPING

According to appellants (Brief, page 3), "[t]he claims do not stand or fall together, as discussed in more detail below." Appellants, however, failed to explain why any specific claim was separately patentable from another. As set forth in 37 C.F.R. § 1.192(c)(7), emphasis added:

> For each ground of rejection which appellant contests and which applies to a groups of two or more claims, the Board shall select a single claim from the group and shall decide the appeal as to the ground of rejection of the basis of that claim alone unless a statement is included that the claims of the group do not stand or fall together and, in the argument under paragraph (c)(8) of this section, appellant explains why the claims of the group are believed to be separately patentable. Merely pointing out

differences in what the claims cover is not an argument as to why the claims are separately patentable.

Accordingly, the claims will stand or fall together. Since all claims stand or fall together, we limit our discussion to representative independent claim 1. Claims 4-6, and 27-32 will stand or fall together with claim 1. In re Young, 927 F.2d 588, 590, 18 USPQ2d 1089, 1091 (Fed. Cir. 1991).

DISCUSSION

According to the examiner (Answer, page 5), Krivan teach "SLT [Shiga-like toxin] toxemia can lead to hemolytic uremic syndrome." In this regard, the examiner finds (Answer, page 4), Krivan teach "purified high titer, monospecific polyclonal antibodies to Shiga-like toxin obtained by a process of inoculating a bovine animal with a purified active SLT derived from E. coli and selected from the group consisting of SLT I, SLT II, and SLT II[v] and mixtures thereof." In addition, the examiner finds (Answer, page 5), Krivan "teach that 'the present invention provides an antitoxin to one or more SLTs' and that 'a single type of SLT, such as SLT-II or a variant thereof, such as SLT-IIvp, can be injected. This provides polyclonal antibodies that are monospecific to just that type of SLT or variant.'" According to the examiner (Answer, bridging sentence, pages 4-5), Krivan teach "passive immunization of a human or animal against SLT toxemia comprising administering to the human or animal a prophylactically effective amount of the elicited antibody."

Regarding MacLeod, the examiner finds (Answer, page 5), MacLeod teach "passive transfer of neutralizing antibodies and active immunization with a toxoid of purified Shiga-like toxin II variant (SLT-IIv) where used to protect pigs against challenge with SLT-IIv." The examiner recognizes, however, that "[n]either Krivan ... nor MacLeod ... teach of monoclonal human or humanized antibodies.

The examiner relies on Queen and Engleman to make up for the deficiency in Krivan and MacLeod. According to the examiner (id.), Queen teach "methodology for the production of CDR-grafted antibodies...." In this regard, the examiner finds (id.), Queen "set forth that humanized antibodies are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin." Regarding Engleman, the examiner finds (Answer, page 6), Engleman teach "methods for constructing human-human hybrids that secrete human monoclonal antibodies using lymp[h]oblastoid cell lines as fusion partners were well known in the art at the time of the invention."

Based on this evidence, the examiner concludes (id.), "it would have been prima facie obvious to one of ordinary skill in the art to have generated a humanized antibody or a human monoclonal antibody as taught by Queen ... and Engleman..., for use in the method disclosed by Krivan...." According to the examiner (id.), MacLeod provides a reasonable expectation of success by

demonstrating that "protection of pigs against challenge with SLT-IIv when they passively received neutralizing antibodies against SLT-IIv...."[1]

According to appellants (Brief, page 10), Krivan does not disclose or suggest "a human monoclonal antibody that will bind to, and specifically neutralize, a Shiga-like toxin II in humans." We agree that Krivan does not disclose a human monoclonal antibody. The examiner, however, has explained (Answer, page 6) that when Krivan is considered together with Queen and Engleman, a person of ordinary skill in the art would find it prima facie obvious to use a human or humanized antibody "based on the advantages described by Queen ... and Engleman ... (i.e., substantially decreased immunogenicity)." Further, to the extent that appellants would argue that Krivan does not teach a monospecific antibody that will bind to and specifically neutralize a Shiga-like toxin II in humans – we disagree. Krivan expressly discloses (column 10, lines 32-35), "[a] human or animal may be passively immunized against SLT toxemia ... by administering a prophylactically effective amount of the IgG or the antibodies of the invention to the human or animal." As to the antibody taught by Krivan, we direct appellants' attention to Krivan's claims 1, 17 and 18. As the examiner points out (Answer, page 8), Krivan's claims read on each of the SLT I,

[1] According to appellants (Brief, page 6), "[o]nly pigs have been proven to be a good model for humans (it is the only other animal species that naturally develops systemic complications when infected with Shiga toxin-producing E. coli), and therefore a model that allows determination of an effective dosage." Appellants rely (Brief, pages 7-8) on the Glunzer, Leong and Tzipori Declarations to support this assertion. We note appellants reference to the Moorn and Tarr letters attached to the Tzipori declaration which provide additional support for their assertion. In all, we find that the declarations and letters support the examiner's finding (Answer, page 8), MacLeod teaches that pigs have proven to be a good model for humans, and that "[p]assive transfer of neutralizing antibodies against SLT-IIv protected pigs from challenge against SLT-IIv."

SLT II, and SLTIIv toxins individually based upon the phrase "'selected from the group consisting of'" as it appears in Krivan's claim 1. Thus, as we understand it, when considered together, the combination of Krivan, Queen and Engleman, teach a human or humanized monoclonal antibody that will passively immunize against SLT toxemia when prophylactically administered to a human. Accordingly, we are not persuaded by appellants' argument to the contrary.

Appellants assert (Brief, page 10), "Krivan only teaches oral administration of antibody which would not be effective in treating or preventing human disease...." We disagree. According to Krivan (column 11, lines 15-20), "[t]he pharmaceutical preparations of the invention are administered locally, as by injection or topical application, intravenously, orally, intradermally, subcutaneously, intraoccularly, subconjunctively, intramuscularly, and intrathecally. The mode of administration will necessarily depend upon the disease and host involved." Accord, Answer, page 10. Accordingly, we are not persuaded by appellants' argument.

According to appellants (Brief, pages 10-11), the SLT-IIv toxin taught by MacLeod is similar to but not the same as SLT-II that causes disease in humans. Accordingly, appellants conclude (Brief, page 11), "if one followed the teaching of MacLeod, one might be able to treat the edema disease of pigs, but it would not help prevent or treat human disease." In our opinion, appellants miss the point. Appellants admit (Brief, pages 6-7), "[o]nly pigs have been proven to be a good model for humans (it is the only other animal species that naturally develops systemic complications when infected with Shiga toxin-producing E.

coli." Appellants' rely on several Declarations in support of this assertion. As the examiner explains (Answer, page 6), MacLeod demonstrates "protection of pigs against challenge with SLT-IIv when they passively received neutralizing antibodies against SLT-IIv...." Thus MacLeod teach that in the only proven model for humans, passive administration of neutralizing antibodies against SLT-IIv was effective to protect against from challenge with SLT-IIv. In our opinion, this provides a person of ordinary skill in the art with a reasonable expectation of success that when antibodies against SLT-II are administered to humans, as taught by Krivan, they will be effective to treat hemolytic uremic syndrome – a disease caused by SLT-producing E. coli[2]. Accordingly, we are not persuaded by appellants' arguments concerning MacLeod. Further, we are not persuaded by appellants' assertion (Brief, page 13) that Krivan does not "place one of skill in the art with antibodies to SLT-II...." In our opinion, Krivan places antibodies to SLT-I, SLT-II and SLT-IIv in the hands of a person of ordinary skill in the art. See e.g., Krivan, examples I-VI, particularly, example II (column 16, lines 32-35), "[t]hree separate cows were injected with purified toxin, one with each major antigenic class of toxin (SLT-I, SLT-II, or SLT-IIv)." Antibodies to each of SLT-1, SLT-II and SLT-IIv, where then isolated from the respective cows. Accordingly, we are not persuaded by appellants' argument to the contrary.

[2] See Krivan, column 1, lines 47-52.

According to appellants (Brief, bridging paragraph, pages 11-12), Queen does not "incorporate the use of an intact 'immune system' to produce ... humanized monoclonal antibodies." We must admit that appellants' point is less than clear. Nevertheless, appellants' claim 1 reads on "humanized antibodies," No claim presented for our review requires "an intact immune system to produce humanized monoclonal antibodies." Queen teaches the production of humanized antibodies. Taken together with Krivan, the combination of Queen and Krivan teach a humanized SLT-II antibody. Accordingly, we are not persuaded by appellants' argument.

According to appellants (Brief, page 15), "[t]here is no teaching in the cited references, singly or in combination, of an effective dosage to prevent or treat hemolytic uremic syndrome in a human." Initially, we note that none of appellants' claims recites a particular dosage. Instead, appellants' claims require that the dosage be an amount that is effective to treat or prevent hemolytic uremic syndrome. See e.g., appellants' claim 1. As the examiner points out (Answer, page 8), Krivan discloses at column 10, lines 48-54,

> [t]he actual amount of IgG or antibodies to be administered for a prophylactic or therapeutic effect will depend upon the particular disorder being treated and the size and/or age of the human or animal. Such dosages will be readily determinable by those of ordinary skill in the art, given the teachings contained herein. The usual dose range would be 100 mg to 5 gm of immunoglobulin.

Accordingly, we disagree with appellants' assertion that the references relied upon by the examiner do not teach an effective dosage to prevent or treat hemolytic uremic syndrome in a human. We are also not persuaded by

appellants' assertion (Brief, page 16) that the range of 100 mg to 5 gm taught by Krivan "greatly exceeds the amount that would be parenterally administered to a human child." No claim on appeal is limited to the treatment of a "human child." We note that while infants, young children and the elderly are most susceptible, there is no evidence on this record that infants, young children and the elderly are the only humans affected by hemolytic uremic syndrome. See e.g., Krivan, column 1, lines 53-55.

On reflection, we find that the examiner has presented the evidence necessary to establish a prima facie case of obviousness. Accordingly, the burden of coming forward with evidence or argument was properly shifted to appellants. In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). Appellants, however, failed to carry their burden. Accordingly, we affirm the rejection of claim 1 under 35 U.S.C. § 103, as being unpatentable over the combination of Krivan, MacLeod, Queen and Engleman. As discussed supra claims 4-6 and 27-32 fall together with claim 1.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

AFFIRMED

Toni R. Scheiner
Administrative Patent Judge

Donald E. Adams
Administrative Patent Judge

Lora M. Green
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

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0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.6.3125–3130.2003

Stx2-Specific Human Monoclonal Antibodies Protect Mice against Lethal Infection with *Escherichia coli* Expressing Stx2 Variants

Abhineet S. Sheoran,[1] Susan Chapman,[1] Pradeep Singh,[2] Arthur Donohue-Rolfe,[1] and Saul Tzipori[1]*

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Shiga toxin-producing *Escherichia coli* (STEC) strains are responsible for causing hemolytic-uremic syndrome (HUS), and systemic administration of Shiga toxin (Stx)-specific human monoclonal antibodies (HuMAbs) is considered a promising approach for prevention or treatment of the disease in children. The goal of the present study was to investigate the ability of Stx2-specific HuMAbs to protect against infections with STEC strains that produce Stx2 variants. Dose-response studies on five HuMAbs, using the mouse toxicity model, revealed that only the three directed against the A subunit were protective against Stx2 variants, and 5C12 was the most effective among the three tested. Two HuMAbs directed against the B subunit, while highly effective against Stx2, were ineffective against Stx2 variants. In a streptomycin-treated mouse model, parenteral administration of 5C12 significantly protected mice up to 48 h after oral bacterial challenge. We conclude that 5C12, reactive against the Stx2 A subunit, is an excellent candidate for immunotherapy against HUS and that antibodies directed against the A subunit of Stx2 have broad-spectrum activity that includes Stx2 variants, compared with those directed against the B subunit.

Infection with Shiga toxin-producing *Escherichia coli* (STEC) strains is associated with hemolytic-uremic syndrome (HUS) (2, 24, 27), the leading cause of acute renal failure in young children (11). Shiga toxins (Stx) are cytotoxins and are major virulence factors of STEC. There are two immunologically distinct Stx types, known as Stx1 and Stx2, of which Stx1 is largely homogeneous, whereas the Stx2 group is highly heterogeneous and consists of at least 10 Stx2 gene variants (10, 14, 23, 31, 32, 37, 41, 42, 50). Stx2 is the most prevalent genotype identified in STEC isolated from patients with HUS (9, 40), and Stx2c is the most common Stx2 variant associated with HUS (9). Stx2 variants other than Stx2c are found frequently in asymptomatic STEC carriers but can often cause uncomplicated diarrhea (9) and rarely cause HUS (14, 33, 38, 45). Stx2f, identified in *E. coli* from pigeons, has been identified only once in humans, in a patient with diarrhea in Canada (10). In addition to pathogenicity to humans, Stx2 is more toxic to mice and piglets than Stx1. Stx2 is about 400 times more lethal to mice than Stx1 when administered systemically (44). STEC strains producing Stx2 alone cause more-severe neurologic symptoms in gnotobiotic piglets than STEC strains producing both Stx1 and Stx2, or Stx1 alone (8).

The nomenclature of Stx2 is confusing; Stx2vha and Stx2vhb (18), which are closely related to Stx2c (42), were originally identified as vtx2ha and vtx2hb (14). They were later shown to be activated by intestinal mucus (21) and named Stx2d (22). However, the Stx2d we refer to in the present study is the Stx2d cluster defined by Pierard et al. (37), which comprises Stx2d-OX3a (32), Stx2d-Ount (37), and Stx2d-O111 (33). An Stx molecule consists of a monomeric A subunit and a pentameric B subunit. Among STEC strains with different Stx2 variants, generic differences in either the A or the B subunit or in both often confer antigenic and functional differences. The amino acid sequence identities of the A subunits of variants Stx2c (42), Stx2vha (14), Stx2vhb (14), Stx2d-OX3a (32), Stx2d-Ount (37), Stx2d-O111 (33), Stx2e (39), and Stx2f (47) with the A subunit of Stx2 are 100, 99, 99, 95, 93, 95, 94, and 71%, respectively. For the B subunit the amino acid sequence homologies are 96, 96, 96, 87, 88, 88, 87, and 82%, respectively.

The two current therapeutic approaches for HUS involve neutralization of Stx either in the gut or in the bloodstream. The two approaches attempted for Stx inactivation in the gut are (i) utilization of glycoconjugate polymers carrying Pk-trisaccharide sequences that serve as a receptor of Stx (1, 4, 5, 17) and (ii) use of recombinant bacteria displaying a Stx-specific glycolipid (globotriose or globotetraose) receptor (29, 30). We believe that systemic administration of Stx-specific neutralizing antibodies is currently the most promising approach for prevention or treatment of Stx-mediated systemic complications, including HUS (7) and edema disease in pigs (15). Murine Stx1- and Stx2-specific monoclonal antibodies (MAbs) have been shown to neutralize both toxins in vitro and in vivo (13, 28, 43). However, murine MAbs are not considered appropriate for human use. Reshaping of a murine antibody against Stx2 into a humanized form has recently been shown to completely protect mice against a lethal challenge with STEC when the antibody is administered within 24 h after infection (51). The disadvantage of a humanized antibody is that it still has mouse components and reduced affinity (12).

Mukherjee et al. have recently generated a panel of 50 human MAbs (HuMAbs) against Stx1 and Stx2 in transgenic

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mice (25, 26), from which we have selected a panel of 5 Stx2-specific HuMAbs that were shown to be highly protective for piglets, even when administered 12 h after an oral challenge with Stx2-producing STEC. In the present study, we used the mouse toxicity model (13, 25, 26, 28, 43) and the streptomycin-treated mouse model of STEC infection (22, 48, 49) to investigate the abilities of these five HuMAbs to protect against Stx2 variants.

MATERIALS AND METHODS

Bacteria. Enterohemorrhagic *E. coli* (EHEC) O91:H21 strain B2F1, which produces Stx2 variants Stx2vha and Stx2vhb (14), was obtained from the American Type Culture Collection (ATCC 51435). A streptomycin-resistant clone of wild-type B2F1 was produced by serially passaging B2F1 on a Luria-Bertani (LB) broth agar plate containing 30 to 100 μg of streptomycin/ml. EHEC O157 strains E32511 (producing both Stx2c and Stx2) (42) and 93-8059 (producing Stx2 only) were obtained from Andrew MacKenzie (Child and Youth Clinical Trial Network, Ottawa, Canada).

Crude preparation of Stx. A culture supernatant of B2F1 was used as a source of Stx2vha plus Stx2vhb (Stx2vha + Stx2vhb). A colony of wild-type B2F1 grown in 3 ml of LB broth for 7 h in a shaker at 37°C was transferred to a sterile flask containing LB broth at a dilution of 1/500 and incubated overnight in a shaker at 37°C. The culture was centrifuged at 1,750 × *g* for 30 min, and the supernatant was filter sterilized by passage through a 0.22-μm-pore-size filter. Similarly, a culture supernatant of E32511 was used as a source of Stx2c and Stx2, and a culture supernatant of 93-8059 was used as a source of Stx2.

Stx2-specific HuMAbs. Production of 37 hybridomas secreting Stx2-specific HuMAbs has been described elsewhere (25). Three HuMAbs against the A subunit (3E9, 2F10, and 5C12) and two against both the A and B subunits (5H8 and 6G3) have been shown to be the most efficient at neutralizing Stx2 in vitro and in vivo (25). These were selected for the present study. All five HuMAbs were of the human immunoglobulin G1(κ) [IgG1(κ)] isotype. HuMAb-containing ascites fluid was prepared by injecting hybridoma cells into the peritoneal cavities of pristane (Sigma-Aldrich Co.)-primed ICR SCID mice (Taconic, Germantown, N.Y.).

Quantitation of Stx2-specific HuMAbs by ELISA. The human IgG1(κ) concentration of each HuMAb in mouse ascites was measured by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated overnight at 4°C with 100 μl of the mouse MAb JDC-1 (IgG1 isotype) against human IgG1 (BD PharMingen) at 5 μg/ml. Plates were washed with phosphate-buffered saline–0.05% Tween 20 (PBS-T) and blocked with 100 μl of 2% nonfat dry milk powder in PBS-T/well at 37°C. After a wash, ascites samples diluted 1:100 in PBS-T were serially diluted twofold in duplicate rows of the plate (100 μl/well). A human IgG1(κ) (Sigma, St. Louis, Mo.) standard was similarly titrated on each plate from a starting concentration of 1 μg/ml. The plates were incubated at 37°C for 1 h and washed again. Horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotech, Birmingham, Ala.), which was affinity purified and cross-adsorbed with human IgA, IgM, and IgD, was added at 100 μl/well at a dilution of 1/1,000. After incubation at 37°C for 1 h and a wash, plates were developed with a substrate solution (0.2% *o*-phenylenediamine–0.05% hydrogen peroxide in citric acid-phosphate buffer [pH 5.0]). The chromogenic reaction was stopped by using 50 μl of 2 M sulfuric acid, and absorbance was read at 490 nm. By using the linear portion of the IgG1(κ) standard curve, the total IgG1(κ) content of each HuMAb in ascites was determined and expressed as milligrams or micrograms of IgG1(κ) per milliliter of ascites fluid.

HeLa cell cytotoxicity neutralization assay. An in vitro HeLa cell cytotoxicity assay was used to evaluate the ability of each HuMAb to neutralize the toxic effects of Stx2vha + Stx2vhb exerted against HeLa cells. Briefly, HeLa cells were plated at 1.4×10^4/well on 96-well plates in McCoy's 5A medium (Mediatech, Inc., Herndon, Va.) containing 10% fetal bovine serum (Harlan Bioproducts for Science, Inc., Madison, Wis.) and incubated overnight at 37°C under 5% CO_2. A culture supernatant of B2F1 containing Stx2vha + Stx2vhb was titrated on HeLa cells to determine a dilution that killed ~70% of HeLa cells. Dead cells were removed by a wash with PBS, and crystal violet was used to stain viable cells (16). A mixture of the culture supernatant at a dilution that killed ~70% of HeLa cells and the HuMAb (5 μg/ml) or IgG1(κ) (5 μg/ml) as an isotype control (Sigma) was preincubated for 1 h at 37°C under 5% CO_2, then added to the cells, and incubated overnight at 37°C under 5% CO_2. A rabbit anti-Stx2 serum at a dilution of 1/400 was used as a positive control. The assay was similarly performed with a culture supernatant of EHEC O157 strain 93-8059 (a Stx2 producer), which served as another control. Plates were developed by crystal violet staining, and absorbance (optical density) was read at 690 nm. The percent neutralization of Stx2vha-, Stx2vhb-, and Stx2-mediated HeLa cell cytotoxicity by the HuMAb was then determined. Similarly, the HeLa cell cytotoxicity neutralization assay was performed utilizing culture supernatants of E32511 and 93-8059.

Mouse toxicity model. The mouse toxicity model (13, 25, 26, 28, 43) was used to determine the most efficacious HuMAb for neutralizing the effects of Stx2vha + Stx2vhb in vivo. Dose-response studies were performed with groups of 10 3- to 4-week-old female Swiss Webster mice (Taconic) to determine the amount of Stx2vha + Stx2vhb in the B2F1 culture supernatant required to induce 100% mortality in untreated animals. A volume of 160 μl of the B2F1 culture supernatant was sufficient (data not shown). The efficacies of HuMAbs were evaluated by administering every Stx2-specific HuMAb intraperitoneally (i.p.) to each of 10 3- to 4-week-old Swiss Webster mice at a dose of 1.25, 2.5, 5, 10, or 20 μg/mouse in 200 μl of PBS, followed 18 h later by i.p. administration of 160 μl of the B2F1 culture supernatant. A control group of 10 mice received human myeloma IgG1(κ) (20 μg/mouse; Sigma), and another control group received 200 μl of PBS alone. Both control groups were also challenged with 160 μl of the B2F1 culture supernatant. Mice were observed twice daily for survival.

Streptomycin-treated mouse model of STEC infection. A streptomycin-treated mouse model of STEC infection (22, 48, 49) was used to investigate the time-dependent efficacy of the most efficacious Stx2-specific HuMAb following infection with B2F1. Four-week-old DBA/2J mice were given drinking water containing 5 mg of streptomycin/ml for 24 h and were then denied food for 12 to 18 h. The mice received 10^{10} CFU of a streptomycin-resistant clone of B2F1 (0.1 ml) in 20% sucrose solution by oral administration. The animals were then permitted access to food and water containing 5 mg of streptomycin/ml ad libitum for the duration of the experiment (12 days). The efficacy of the most efficacious Stx2-specific HuMAb, 5C12, was tested following i.p. administration at a dose rate of 2.1 mg/kg of body weight following 0, 12, 24, 48, and 72 h of oral infection with 10^{10} CFU of B2F1. A group of 10 mice was used for each time point. A control group of 10 mice received human myeloma IgG1(κ) (30 μg/mouse injected i.p.; Sigma) at 0 h following infection with B2F1. Mice were observed three times per day for survival.

Immunoblotting. In addition to in vitro and in vivo neutralization of Stx2vha + Stx2vhb by HuMAbs, the reactivity of each HuMAb with Stx2vha + Stx2vhb was determined by immunoblotting. Stx2, purified as described elsewhere (6), and a 55-fold-concentrated culture supernatant of B2F1 as a source of Stx2vha + Stx2vhb were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred to a 0.2-μm-pore-size nitrocellulose membrane (Bio-Rad Laboratories, Richmond, Calif.). After transfer, the membrane was blocked with 5% nonfat dry milk powder in PBS-T at room temperature for 1 h, washed, and incubated with each HuMAb (2.5 μg/ml of PBS-T) at room temperature for 1 h. Human IgG1(κ) (Sigma) was used as a control. After a wash, strips were incubated with horseradish peroxidase-conjugated goat anti-human IgG (Southern Biotech) at a dilution of 1/1,000 for 1 h at room temperature and then washed and developed with the 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase-substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.).

Statistical analysis. The grouped survival data were analyzed by the Mantel-Cox test and by using the PROC Freq procedure of SAS statistical software. Resulting *P* values of <0.05 were considered significant.

RESULTS

Reactivity in immunoblotting. HuMAbs 5C12, 3E9, and 2F10 reacted with the A subunits of Stx2 and Stx2vha + Stx2vhb (Fig. 1). HuMAbs 6G3 and 5H8 reacted strongly with the B subunit and mildly with the A subunit of Stx2 but did not react with any of the subunits of Stx2vha + Stx2vhb.

Neutralization of Stx2vha- and Stx2vhb-mediated HeLa cell cytotoxicity. Each of the Stx2-specific HuMAbs was effective at neutralizing the activity of Stx2 present in the culture supernatant of 93-8059; however, differences in relative potency were observed (5H8 and 6G3 showed the highest potency, followed, in descending order, by 5C12, 2F10, and 3E9) (Fig. 2). Similarly, consistent relative differences were observed among the Stx2 A-subunit-specific HuMAbs with respect to

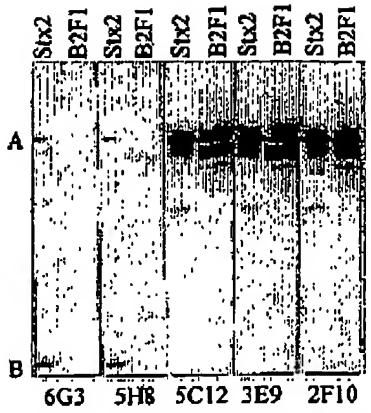

FIG. 1. Immunoblot reactivities of HuMAbs with Stx2 and Stx2 variants. Lanes Stx2 and lane B2F1 represent purified Stx2 and a concentrated culture supernatant of B2F1 containing Stx2 variants (Stx2vha and Stx2vhb), respectively. HuMAbs used to react with the Stx are given below the blot. Bands A and B represent the A and B subunits, respectively.

neutralization of Stx2vha + Stx2vhb present in the culture supernatant of B2F1 and neutralization of Stx2c present in the culture supernatant of E32511 (5C12 and 2F10 showed approximately equal potencies, while that of 3E9 was lower). In contrast, the B-subunit-specific HuMAb 5H8 did not neutralize Stx2vha + Stx2vhb or Stx2c, and 6G3 neutralized them at very low levels. Although 5C12 neutralized Stx2 completely, residual cytotoxicity was observed with Stx2vha + Stx2vhb and Stx2c; this might have been due to the presence of toxic factors other than Stx2 variants in the culture supernatants of B2F1

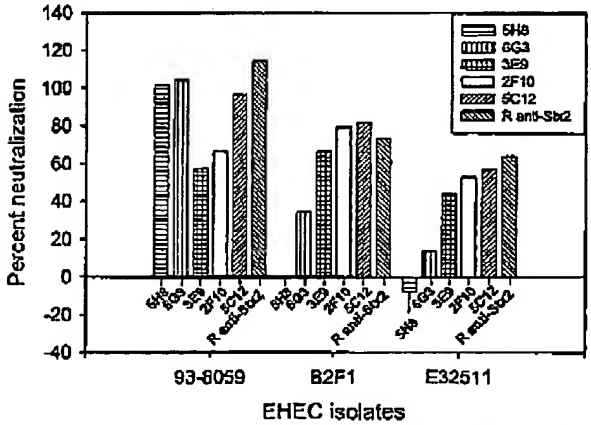

FIG. 2. Neutralization of HeLa cell cytotoxicity mediated by Stx2 (produced by 93-8059), Stx2vha + Stx2vhb (produced by B2F1), and Stx2 plus Stx2c (produced by E32511) by Stx2-specific HuMAbs. The B-subunit-specific HuMAbs 5H8 and 6G3 neutralized Stx2 completely. However, 5H8 did not neutralize Stx2vha, Stx2vhb, or Stx2c, and 6G3 neutralized them mildly. The A-subunit-specific HuMAb 5C12 and rabbit anti-Stx2 serum (R anti-Stx2) strongly neutralized all Stx types. The other A-subunit-specific HuMAbs, 2F10 and 3E9, were also very effective.

and E32511, since the rabbit anti-Stx2 serum also neutralized Stx2 completely but did not completely neutralize the Stx2 variants.

Neutralization of Stx2vha + Stx2vhb in vivo. Considering the identical in vitro neutralization patterns of the Stx2-specific HuMAbs against Stx2c and Stx2vha + Stx2vhb (Fig. 2), further studies to examine the relative potency of each HuMAb in vivo were performed only against Stx2vha + Stx2vhb, by utilizing the mouse toxicity model. At each dose, Stx2 A-subunit-specific HuMAbs 5C12, 2F10, and 3E9 significantly protected mice, as evidenced by comparison with the PBS control (average survival, 2.30 ± 0.35 days) and the HuMAb IgG1(κ) control (average survival, 2.35 ± 0.34 days) ($P < 0.0001$) (Fig. 3). In contrast, Stx2 B-subunit-specific HuMAbs 5H8 and 6G3 did not protect mice significantly at any dose level. HuMAbs 2F10 and 3E9 exhibited very similar dose-dependent effects on relative average survival; they did not differ significantly from each other at any dose level except 10 μg/mouse ($P < 0.0001$). In contrast, 5C12 did not show dose dependency; it protected 90% of the mice even at the lowest dose administered (1.25 μg/mouse). 5C12 provided better protection than 2F10 and 3E9, differing significantly from them at all dose levels except for 3E9 at doses of 10 and 2.5 μg/mouse. At the lowest dose (1.25 μg/mouse) tested, 5C12 was far superior to 2F10 and 3E9 ($P < 0.0001$).

Time-dependent efficacy of 5C12 in B2F1-infected mice. To test for the time-dependent efficacy of 5C12, mice were orally infected with 10^{10} CFU of B2F1, and HuMAb 5C12 was administered at 0 to 72 h after infection (Fig. 4). All control mice infected and treated i.p. at the same time with control human IgG1(κ) died, with an average survival time of 6 days. In contrast, 5C12 administered 0, 12, 24, or 48 h following infection protected 80% ($P = 0.0001$), 70% ($P = 0.0002$), 90% ($P < 0.0001$), or 60% ($P = 0.001$) of the mice, respectively (Fig. 4). However, 5C12 administered 72 h following infection protected only 20% of the mice, which was not a significant effect.

DISCUSSION

The main goals of the present study were (i) to identify the most effective Stx2-specific HuMAb by using the mouse toxicity model and (ii) to determine the protective ability of the selected HuMAb against Stx2c, the most prevalent Stx2 variant associated with HUS (9), by using the streptomycin-treated mouse model of oral STEC infection (22, 48, 49). Like others (18), we were unsuccessful in adapting the streptomycin-treated mouse model for strain E32511, which produces both Stx2 and Stx2c (data not shown). Consequently, we have used strain B2F1, described by other investigators (22, 48, 49, 51), which expresses both Stx2vha and Stx2vhb (14). B2F1 was ideal because the B subunits of Stx2c, Stx2vha, and Stx2vhb are identical and differ from the B subunit of Stx2 by 2 amino acids (14, 42). In addition, unlike the A subunit of Stx2c, which is identical to that of Stx2, the A subunits of Stx2vha and Stx2vhb differ by 3 amino acids from that of Stx2. Therefore, testing Stx2vha and Stx2vhb against our Stx2 A- and B-subunit-specific HuMAbs determined not only the influence of amino acid differences in the B subunit, but also that for the A subunit, on the relative protective abilities of the HuMAbs. Moreover,

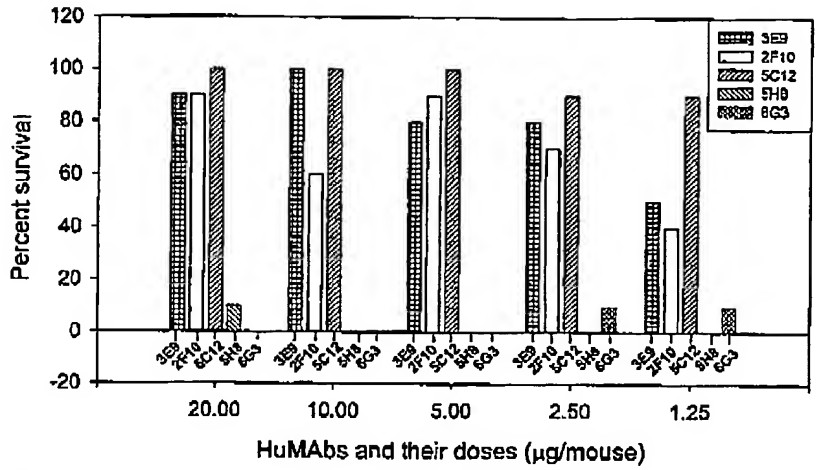

FIG. 3. Percent survival of mice given 20, 10, 5, 2.5, or 1.25 μg of HuMAb 3E9, 2F10, 5C12, 5H8, or 6G3 i.p., followed 18 h later with a 100% lethal dose of the Stx2vha + Stx2vhb-containing culture supernatant of EHEC isolate B2F1, also given i.p. Stx2 A-subunit-specific HuMAbs 5C12, 2F10, and 3E9 significantly protected mice relative to the PBS control (average survival, 2.30 ± 0.35 days) and the HuMAb IgG1(κ) control (average survival, 2.35 ± 0.34 days) ($P < 0.0001$). HuMAbs 5H8 and 6G3 did not protect mice. HuMAbs 2F10 and 3E9 exhibited very similar dose-dependent effects on relative average survival; they did not differ significantly from each other at any dose level except 10 μg/mouse ($P < 0.0001$). In contrast, 5C12 did not show dose dependency; it protected 90% of the mice even at the lowest dose administered (1.25 μg/mouse). At the lowest dose (1.25 μg/mouse) tested, 5C12 was far superior to 2F10 and 3E9 ($P < 0.0001$).

STEC strains producing Stx2vha + Stx2vhb have also been associated with HUS (14).

We first evaluated the neutralizing abilities of the five selected HuMAbs against Stx2 variants (Stx2c and Stx2vha + Stx2vhb) in vitro by HeLa cell cytotoxicity neutralization assay, followed by dose-response studies in the mouse toxicity model (13, 25, 26, 28, 43). Of the five HuMAbs tested (three A-subunit and two B-subunit specific), 5C12 was the most effective, and therefore it was selected for further evaluation in the streptomycin-treated mouse model of infection (22, 48, 49).

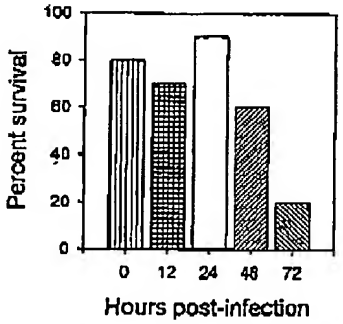

FIG. 4. Percent survival of mice orally infected with Stx2vha + Stx2vhb-producing B2F1 and given 5C12 i.p. at a dose of 2.1 mg/kg of body weight at various times postinfection. All control mice infected and treated i.p. at the same time with control human IgG1(κ) (2.1 mg/kg of body weight) died, with an average survival time of 6 days. In contrast, 5C12 administered 0, 12, 24, or 48 h following infection protected 80% ($P = 0.0001$), 70% ($P = 0.0002$), 90% ($P < 0.0001$), or 60% ($P = 0.001$) of the mice, respectively. However, 5C12 administered 72 h following infection protected only 20% of the mice, which was not significant.

5C12 was administered at various time points after bacterial challenge, since treatment of patients with STEC infection is expected to occur after exposure to infection, at the onset of bloody diarrhea. Studies with piglets have already shown that these HuMAbs are protective even when given after an oral bacterial challenge with Stx2-producing STEC (25). This is the first report, however, which shows that administration of a specific HuMAb against Stx2 (5C12 at 2.1 mg/kg) can significantly protect mice when given as long as 48 h after bacterial challenge. In contrast, a study using the same mouse model and strain B2F1 has shown that the Stx2-specific humanized MAb TMA-15, given at a dose of 1.0 mg/kg, protects mice when given as long as 24 h after bacterial challenge (51). It is possible that the differences in length of protection afforded by 5C12 and TMA-15 are due to differences in their respective affinities. Although concentration of Stx2 variants in the blood were not determined in the present study, Yamagami et al. (51) have reported that serum Stx2 variant levels are highest in mice at 48 h after STEC infection (51). This suggests that 5C12 can significantly protect mice even when the maximum levels of Stx2 variants are present in the bloodstream. The time window of 48 h for immunotherapeutic intervention has direct implications for children at risk of developing HUS (e.g., those presenting with bloody diarrhea or excreting STEC) and for individuals in contact with them. The development of rapid and sensitive diagnostic methods has made it possible to detect STEC infections almost a week before symptoms of HUS become apparent (34, 35).

The three amino acid differences between the A subunits of Stx2vha and Stx2vhb, on the one hand, and the A subunit of Stx2, on the other (14), did not significantly affect the binding of any of the Stx2 A-subunit-specific HuMAbs; all of them neutralized Stx2vha + Stx2vhb both in vitro and in vivo. How-

ever, the Stx2 B-subunit-specific HuMAbs (5H8 and 6G3) failed to neutralize Stx2vha + Stx2vhb, suggesting that one or both of the amino acid changes in the B subunits of Stx2vha and Stx2vhb considerably affected the neutralizing capabilities of 5H8 and 6G3. Since the B subunits of Stx2vha and Stx2vhb are identical to the Stx2c B subunit, 5H8 and 6G3 also failed to neutralize Stx2c in vitro. However, Stx2 A-subunit-specific HuMAbs neutralized Stx2c in vitro, because Stx2 and Stx2c have identical A subunits. The failure of 6G3 and 5H8 to neutralize Stx2c in vitro and their stronger immunoblot-reactivity with the B subunit than with the A subunit of Stx2 unequivocally show that the neutralization activities of these two HuMAbs are due to their binding with the B subunit and not the A subunit.

Given that STEC can produce any combination of Stx1, Stx2, and/or Stx2c (9), an ideal therapeutic formulation should, in our view, include HuMAbs specific for Stx1, Stx2, and Stx2c. Mukherjee et al. have recently reported production of protective Stx1-specific HuMAbs (26) for inclusion in such a formulation. Since it appears from this study that A-subunit-specific Stx2 antibodies display inhibitory activity against Stx2c as well, the selection of 5C12 combined with an effective Stx1-specific HuMAb, described in an earlier study (25), could provide broad-spectrum protection against Stx1, Stx2, and Stx2c. However, the efficacy of 5C12 needs to be further investigated in the orally infected piglet model, since piglets are the only species in addition to humans that are naturally susceptible to the systemic effects of Stx produced by *E. coli* strains that proliferate in the gastrointestinal tract (19, 20), with characteristic attachment-and-effacement lesions (36, 46), which are absent in the mouse (18). The mouse model is also less susceptible to Stx, as judged by the amount of toxin required to cause death compared to that for the piglet (3, 26) and presumably for children. The mouse infection model, however, is useful for screening and evaluation, because it is genetically uniform, available in large numbers, easy to manipulate, requires smaller amounts of reagents, and is less expensive and less labor-intensive. The piglet model, on the other hand, is more appropriate for preclinical evaluation of formulations and for validation, including determination of the likely effective therapeutic dose for humans.

We conclude that 5C12, which is reactive against the Stx2 A subunit, is an excellent candidate for immunotherapy against HUS and that antibodies directed against the A subunit of Stx2, as opposed to those directed against the B subunit, have broad-spectrum activity that includes Stx2 variants.

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